# INTRACEREBRAL INJECTIONS AND THE GROWTH OF VIRUSES IN THE MOUSE BRAIN

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THE intracerebral injection technique is one that has been widely used in animal virology, especially following the discovery by Theiler (1930) that yellow fever virus was pathogenic for mice when injected by this route. New viruses were thereby isolated and studied in a convenient laboratory animal. In spite of the fact that this technique has been in general use for the past 30 years, little is known of its mechanics, or of the distribution in the brain of the injected material.

When viruses which are injected intracerebrally to mice multiply in the mouse brain and produce a neurological type of illness, they are loosely referred to as "neurotropic" viruses. Less than 5 per cent of the cells in the brain, however, are neurones (Nurnberger and Gordon, 1957), and since the cells which support virus growth can now be identified in sections by the fluorescent antibody technique, a re-examination of the "neurotropism" of certain viruses has been made using this technique. Experiments on the mechanics of intracerebral injections, and the fate in the brain of the injected material, are also described.

In subsequent papers the adaptation of influenza and vaccinia viruses to mouse brain will be analysed by means of the fluorescent antibody technique.

## MATERIALS AND METHODS

*Mice.*—Multicoloured outbred mice of the Walter and Eliza Hall Institute (Melbourne) strain were used.

*Viruses.*—The Moscow strain of ectromelia (mousepox) was used in the form of an infected liver suspension (Mims, 1959).

The Murray Valley Encephalitis (MVE) virus strain had received a very small number of mouse and egg passages since its isolation, and it was used in the form of an infected mouse brain suspension.

*Titration methods.*—Ectromelia titrations were made by the pock counting method as described in an earlier paper (Mims, 1959). Titres are expressed as pock forming units (pfu) per 0·05 ml. of tissue.

MVE virus titrations were made intracerebrally in adult mice and LD 50 titres calculated by the method of Reed and Muench (1938).

*Fluorescence microscopy.*—Fluorescent antibody staining by the direct method was used in most experiments. The more sensitive indirect method with rabbit antiserum was successfully used for ectromelia virus (Mims, 1959) to confirm results, but many rabbit antisera gave marked non-specific staining of the vascular bed of the brain which could not be prevented by thorough adsorption with either mouse brain or mouse liver powder. Most rabbit antisera also gave slight non-specific staining of nuclei in the brain parenchyma and, although this again could not be prevented by adsorption, the fluorescence it produced was not usually very bright.

Conjugates for the direct method were prepared from fluorescein isothiocyanate (Sylvana Chemical Co., Orange, New Jersey, U.S.A.) and $\gamma$ globulins extracted from sera by $Na_2SO_4$ precipitation.

Vaccinial hyperimmune rabbit serum was used for ectromelia, and monkey immune serum (kindly supplied by Dr. S. G. Anderson) for MVE virus. Tissues were sectioned, stained, examined, and photographed as described in an earlier paper (Mims, 1959).

*Routine histology.*—Brains were fixed in formol saline and stained with haematoxylin and eosin.

## RESULTS

### *Preliminary Experiments with Intracerebral Injections*

#### *The fate in the brain of injected material*

It was shown by Cairns (1950), who used a phage label, that the greater part of an intracerebral inoculum spills over immediately into the blood stream. This was readily confirmed when it was observed that the liver of a mouse blackened a few seconds after an intracerebral injection of Indian ink. A study of the fate of the material which stayed in the brain was made by injecting 0·03 ml. of 10-fold diluted Indian ink intracerebrally in mice, and fixing, sectioning, and staining brain from 10 min. to 48 hr. later. Some unweaned mice were also used, and sections were then cut through the entire head, and through the spinal column. In all cases, the injected material was found to be distributed throughout the subarachnoid space, in many of the Virchow-Robin spaces surrounding blood vessels, and often, but not always in the ventricles (Figs. 1 and 2). In the spinal cord there was ink in the central canal and subarachnoid space, and it extended round the spinal nerve roots as they passed through the vertebral foramina. Indian ink was never observed in the parenchyma of the central nervous system except under the following three circumstances. First, in a few sections an Indian ink-containing fissure was seen, usually joined to a ventricle or subachnoid space and passing in the tissue plane between ganglia or fibre tracts ; such fissures were commoner when larger volumes were injected more rapidly. Second, in some sections the track of the injecting needle was seen, and this contained Indian ink. Third, in the brains removed after 1–2 days glial cells laden with Indian ink were often seen below the pia and ependyma, and it was thought probable that they had migrated into the parenchyma from the subarachnoid spaces or ventricles.

Thus, nearly all the intracerebrally injected material which stays in the brain is deposited throughout the cerebrospinal fluid (CSF) spaces (Fig. 13) and does not come into contact with parenchymatous tissue (Schaeffer and Muckenfuss, 1938). Small amounts may reach the parenchyma along the needle track, or when tissue planes are split by the injection pressure, or by being carried iuto the parenchyma by wandering macrophages (microglia).

#### *The Pressure Used in Intracerebral Injections, and the Volume Injected*

The important experimental work on the passage of material into the blood after its injection into the CSF has been done using carefully controlled physiological injection pressures and volumes. Massive overflows of injected material into the blood do not occur under these circumstances, and it is clear that anatomical barriers must be destroyed to make such overflows possible. A study of the pressures and volumes used in the usual mouse intracerebral injection technique was therefore made.

It was first noted that 0·03 ml., the volume traditionally injected, is a very large volume relative to the volume of the adult mouse brain, which is about

0·4 ml. An equivalent volume injected intracerebrally to a man would be 100 ml.

Injection pressures and rates of flow were investigated by injecting ether anaesthetized mice intracerebrally with saline from a long vertical tube. The tube (0.23 cm. diameter) was connected to a large (21 gauge) needle *via* a tap. The saline level was recorded at the start of each injection, which was continued for $\frac{1}{4}$ or $\frac{1}{2}$ min. One reading was taken from each mouse, and the averaged results from more than 200 mice are recorded in Fig. 14. It can be seen that there is no rapid flow of material into the mouse cranium until the injection pressure is about 150 cm. $H_2O$. The pressures exerted during routine intracerebral injections were found to be of the order of 200–300 cm. $H_2O$, and at such enormous pressures, 20–30 times the normal CSF pressure, the flow is quite rapid. There is no detect-

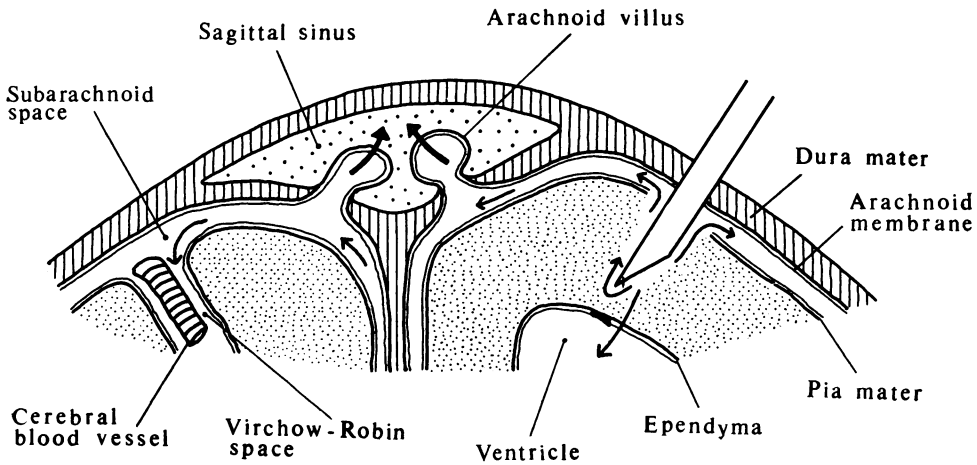



FIG. 13.—Diagram to show the fate of intracerebrally injected material.

able flow and a routine intracerebral injection could not be made, if the pressure is a mere five times the normal CSF pressure.

The skull can be thought of as a more or less rigid box enclosing incompressible material; for every 0·03 ml. which is forced in, 0·03 ml. has to be expelled. Minor adjustments can perhaps be made by the collapse of veins or the redistribution of CSF, but it is known that most of the injected material is in fact forced into the blood stream (see above). It would not be surprising if anatomical barriers were broken down by the use of high injection pressures. It seems likely that the first barriers to break down as the pressure rises, are the arachnoid villi. These relatively thin-walled structures form a barrier between CSF and blood (Fig. 13), and once they have been " blown open " there is a free flow of injected material into the blood. Indeed, from then on the injection becomes an intravenous one, and large volumes can be given. A volume of 0·5 ml. of saline has often been injected intracerebrally to mice and 5 ml. were injected into the one rabbit tested with no ill effects. If large volumes are injected too rapidly, additional anatomical structures are broken down, the inoculum may leak from the nose, and recovery is less probable.

In summary, the grossly unphysiological intracerebral injection methods in common use deposit some of the injected material in the CSF spaces and little or none in nervous tissue. The rest spills over into the blood stream. Injected viruses will necessarily be deposited in the same regions, and a study of the site of growth in the brain of intracerebrally injected viruses will now be reported, and the results considered from this point of view.

### *The Growth of Ectromelia (Mousepox) Virus Following Intracerebral Injection*

*General observations*

Mice dying after extraneural injections of ectromelia virus did not have brain titres of more than $10^{3.0}$ pfu/0·05 ml. brain, even when the brain was traumatized

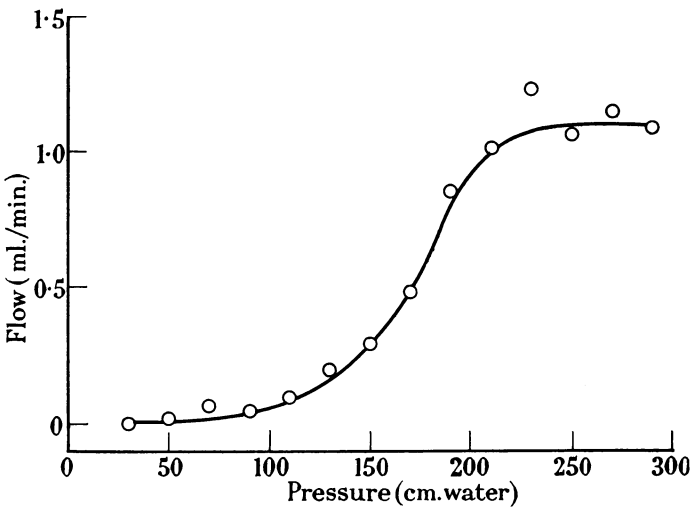



FIG. 14.—The relation between the rate of flow of intracerebrally injected saline and the injection pressure.

at the time of injection. Such titres are attributable to the virus present in the blood in the brain. When, however, large doses of virus are injected intracerebrally into mice, there is growth of virus in the brain, and titres of $10^6$ pfu/0·05 ml. brain are common. Death, which occurs 2–3 days after large intracerebral injections, is preceded by a neurological type of illness. Sick mice tremble, have ruffled fur, are hunched, and may die during the course of convulsions. Convulsions can often be induced by tail twirling and sometimes by merely hanging the mouse upside down. Paralysis never occurs. There is detectable growth of virus in the brain when more than about $10^3$ pfu are injected intracerebrally, in that titres of more than $10^4$ pfu/0·05 ml. brain are reached by the time of death, and the neurological type of illness may occur.

Most of the intracerebrally injected material is forced into the blood stream, so that there is growth of virus in the viscera, just as there is after intravenous injections. Unless they die in a convulsion mice probably die as a result of this

visceral growth of virus rather than as a direct result of growth in the brain, for survival times were the same and equally high liver titres were attained after large doses of virus, whether given intravenously or intracerebrally. It was also shown that the intracerebral injection route is as sensitive as the intravenous one as a test for virus, because a given material gave the same titres (both ID 50 and LD 50) when titrated intracerebrally and intravenously.

An attempt was made to adapt ectromelia to the mouse brain by making 10 brain passages, using large doses at each passage. The brain titres and survival times of injected mice, however, were unchanged. Jahn (1939) also found no increase in the neurotropism of ectromelia virus after 20 brain passages in mice.

*Growth curve experiment*

$10^{7 \cdot 2}$ pfu of ectromelia virus were injected intracerebrally (0·03 ml.) in many mice, and at intervals afterwards 3 mice were killed with ether, their brains pooled, suspended in 6 ml. gelatine saline, and titrated *in ovo*. The time of onset of sick-

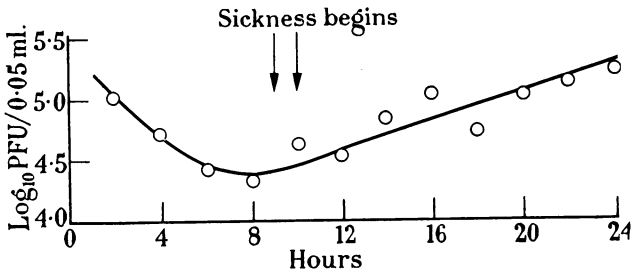



FIG. 15.—The growth of intracerebrally injected ectromelia virus in the brain of mice.

ness was carefully observed. There was a preliminary disappearance of virus in the brain, followed by a gradual rise in titre which began after about 8 hr. (Fig. 15). All mice were well until the ninth hour, when some of them were slightly sick, and most were sick by 12 hr. ; the first mice died after 2 days. Sickness begins much later after large intravenous injections of virus, and is not detectable until at least 24 hr., however much virus is injected (Mims, 1959). This experiment shows that ectromelia virus grows in the mouse brain producing sickness soon after growth first begins.

*The site of growth of virus in the brain*

Brains from mice moribund or very sick 2–3 days after large intracerebral injections of virus were quick-frozen and sections examined by the fluorescent antibody technique. Specific fluorescence was limited to cells lining the CSF spaces, that is, to cells lining the ventricles, subarachnoid space, and Virchow-Robin spaces (Figs. 3–9). The few fluorescent cells seen just below the pia and ependyma were probably glial cells which had migrated into the parenchyma like the Indian ink-laden cells described above. Larger numbers of glial cells were sometimes seen in the parenchyma adjacent to the third ventricle. There was no fluorescence lining the needle tracks even when the intracerebral injection was made with multiple insertions of the needle. Brains examined 7 and 17 hr. after

the injection of virus showed a fainter specific fluorescence, which was, however of similar distribution.

Thus intracerebrally injected ectromelia virus grows mainly in the meninges and ependyma, and not in neural tissue proper. This finding was confirmed in a crude way when brains from moribund infected mice were frozen, and segments cut from meninges and parenchyma with a scalpel. When these segments were suspended in gelatine saline and titrated, it was found that nearly all the virus was in the meningeal material.

Again, the type of sickness in intracerebrally infected mice is meningitic rather than encephalomyelitic, and brains show the pathological changes of meningitis and ependymitis on routine histological examination.

In other fluorescent antibody experiments it was shown that there was the same distribution of antigen in the brains of mice very sick following the intracerebral injection of rabbitpox virus.

### *The Growth of MVE Virus in Mouse Brain*

Since ectromelia had been shown to be meningotropic rather than neurotropic, MVE virus, which might be expected to behave in a more strictly neurotropic fashion, was also studied.

Mice were injected intracerebrally with $10^7$ LD 50 of MVE virus and brains were taken at the time of sickness 4–5 days later, sectioned, and examined by the fluorescent antibody technique. In these brains there was no meningeal or ependymal fluorescence, but there was a bright specific fluorescence of many parenchymal cells (Plate, Figs. 10–12). Some of these cells were almost certainly neurones, although even after haematoxylin and eosin staining it was not possible to say with complete certainty that they were not neuroglia.

Intracerebrally injected MVE virus is mechanically distributed through the CSF spaces, as is Indian ink, and in an attempt to find out how it reaches susceptible parenchymal cells, brains were examined 24 and 48 hr. after large intracerebral injections of virus. No antigen was seen at 24 hr., and small parenchymatous foci were established by 48 hr. In neither case was there antigen in the cells lining the meninges, ependyma, or Virchow-Robin spaces. Another brain was examined 24 hr. after a large amount of virus had been given intracerebrally through five separate injection sites. There was no antigen in the cells lining the needle tracks.

MVE virus thus grows in parenchymal cells after intracerebral injection into mice. Virus probably does not reach susceptible cells by way of the injecting needle track, nor by growing through the ependyma or meninges. Perhaps it is carried through to the parenchyma by wandering macrophages, as was ectromelia virus.

## DISCUSSION

It has been shown that high pressures are needed to inject mice intracerebrally by the usual method. When Indian ink is injected it overflows into the blood stream and is also deposited in the spaces occupied by the CSF. Viruses injected in the same way will necessarily be deposited in the same spaces, and the cells lining these spaces will be the ones immediately available for viruses to grow in. Ectromelia (mousepox) virus, rabbitpox virus and probably many other pox viruses, grow in these cells, producing a meningitis and ependymitis which is

detectable histologically and results in a meningitic type of illness. There is, however, no growth in the brain parenchyma. Buddingh (1938) showed that intracerebrally injected fowlpox virus produced lesions in the meninges, perivascular spaces and ependyma of chicks, with no primary damage to nervous tissue.

It was Levaditi and Nicolau (1923) who first placed certain strains of vaccinia virus in the general category of neurotropic viruses, although Hurst and Fairbrother (1930) reported that monkeys and rabbits injected intracerebrally with neurovaccinia showed a mainly meningeal histological reaction. Hurst and Fairbrother stated that " the virus of vaccinia is not neurotropic in the same sense as that of poliomyelitis ". This conclusion is amply confirmed by the fluorescent antibody studies reported here.

The fluorescent antibody evidence shows that MVE virus grows in the cells in the parenchyma of the brain but that it does not grow in the cells lining the CSF space. The intracerebrally injected virus could cross the CSF-brain barrier and reach parenchymal cells by any of the three routes referred to above for the Indian ink experiments, or by " growing through " ependymal or pial cells as in the case of the NWS strain of influenza virus (Mims, unpublished). There is, however, no detectable growth of virus in the ependyma or meninges, nor in the injecting needle track. The needle track also seems less likely to be important because poliovirus, when carefully injected into the subarachnoid spaces of monkeys with no damage to the underlying tissues, still grows in neurones to produce the typical disease (Hurst, 1932). Virus does not reach the parenchyma along tissue planes split open by the injected material because it was found that there was growth as usual in the brain when a virus-contaminated needle by itself was inserted intracerebrally. This leaves open the possibility that migrating macrophages carry MVE virus across the CSF-brain barrier.

## EXPLANATION OF PLATES

FIG. 1.—Haematoxylin and eosin stained section of the brain of a mouse 10 min. after the intracerebral injection of Indian ink. The ink is restricted to the subarachnoid space at the surface of the brain. ( × 33).

FIG. 2.—Similar section containing the hippocampus and neighbouring structures with the Indian ink again restricted to the subarachnoid space. ( × 13).

FIG. 3.—Fluorescein labelled ectromelia antigen in mouse brain.

FIG. 4.—Same field stained with haematoxylin and eosin, to show the extensive areas of antigen-free parenchyma, and the localization of antigen to the subarachnoid space of a nearby sulcus. ( × 10).

FIG. 5.—Fluorescein labelled ectromelia antigen in mouse brain.

FIG. 6.—The same field stained with haematoxylin and eosin, showing that antigen is restricted to the subarachnoid space of the meninges, although a few fluorescent cells are seen in the adjacent parenchyma. This mouse received an intracerebral injection of Indian ink shortly after the virus injection. Blood covers the meninges, and there is a large vein in the subarachnoid space. ( × 60).

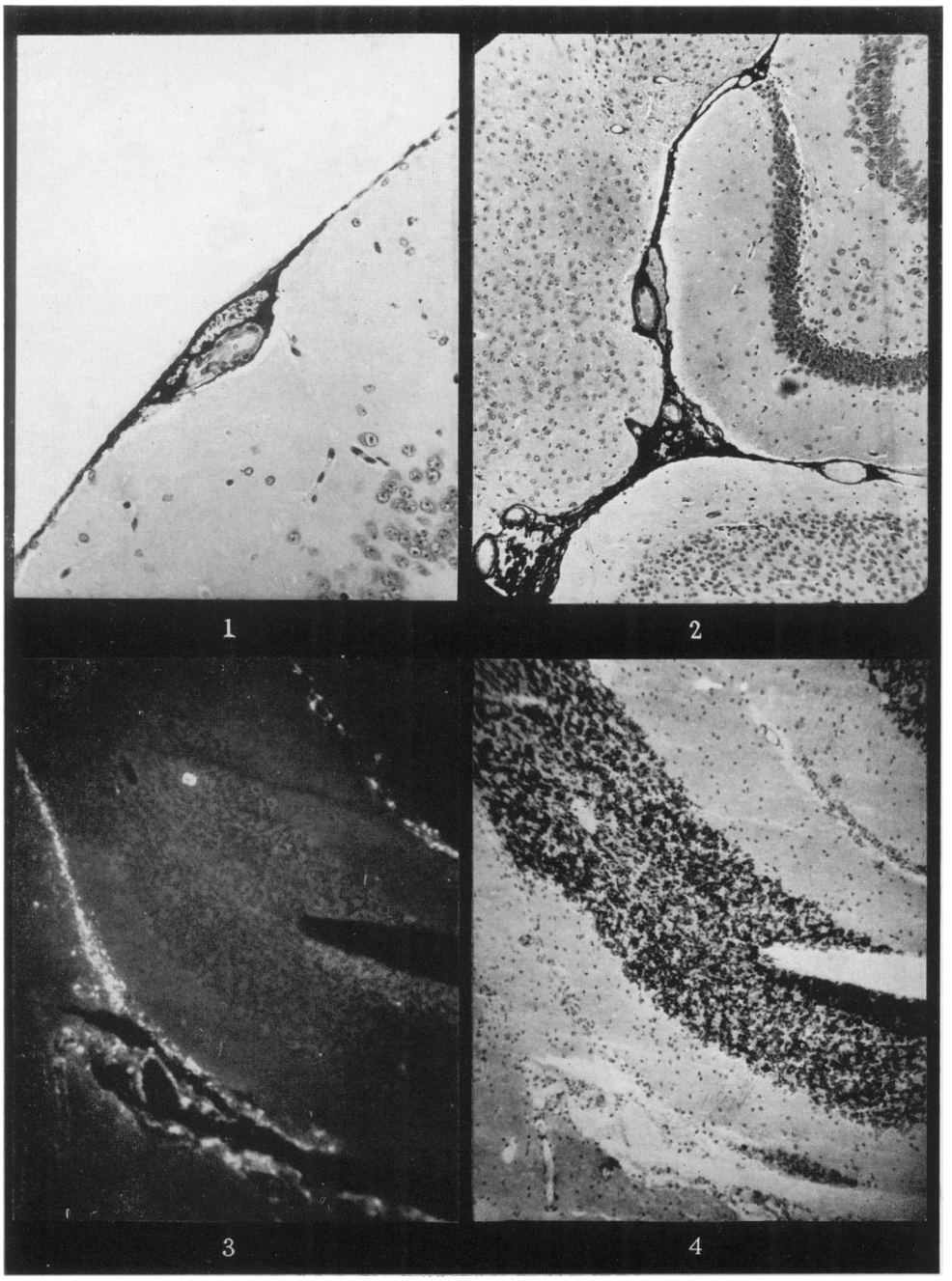
FIG. 7.—Fluorescein labelled ectromelia antigen in mouse brain.

FIG. 8.—The same field stained with haematoxylin and eosin. Antigen is present only in the ependymal cells lining the ventricle. ( × 150).

FIG. 9.—Fluorescein labelled ectromelia antigen in mouse brain, present in the Virchow-Robin spaces surrounding blood vessels. ( × 80).

FIG. 10.—Fluorescein labelled MVE antigen in mouse brain. The meninges contain no antigen, although a small focus of antigen-containing cells is present in the neighbouring parenchyma. ( × 40).

FIGS. 11 and 12.—Fluorescein labelled MVE antigen in mouse brain. Parenchymatous cells contain antigen which is limited to the cytoplasm. ( × 700).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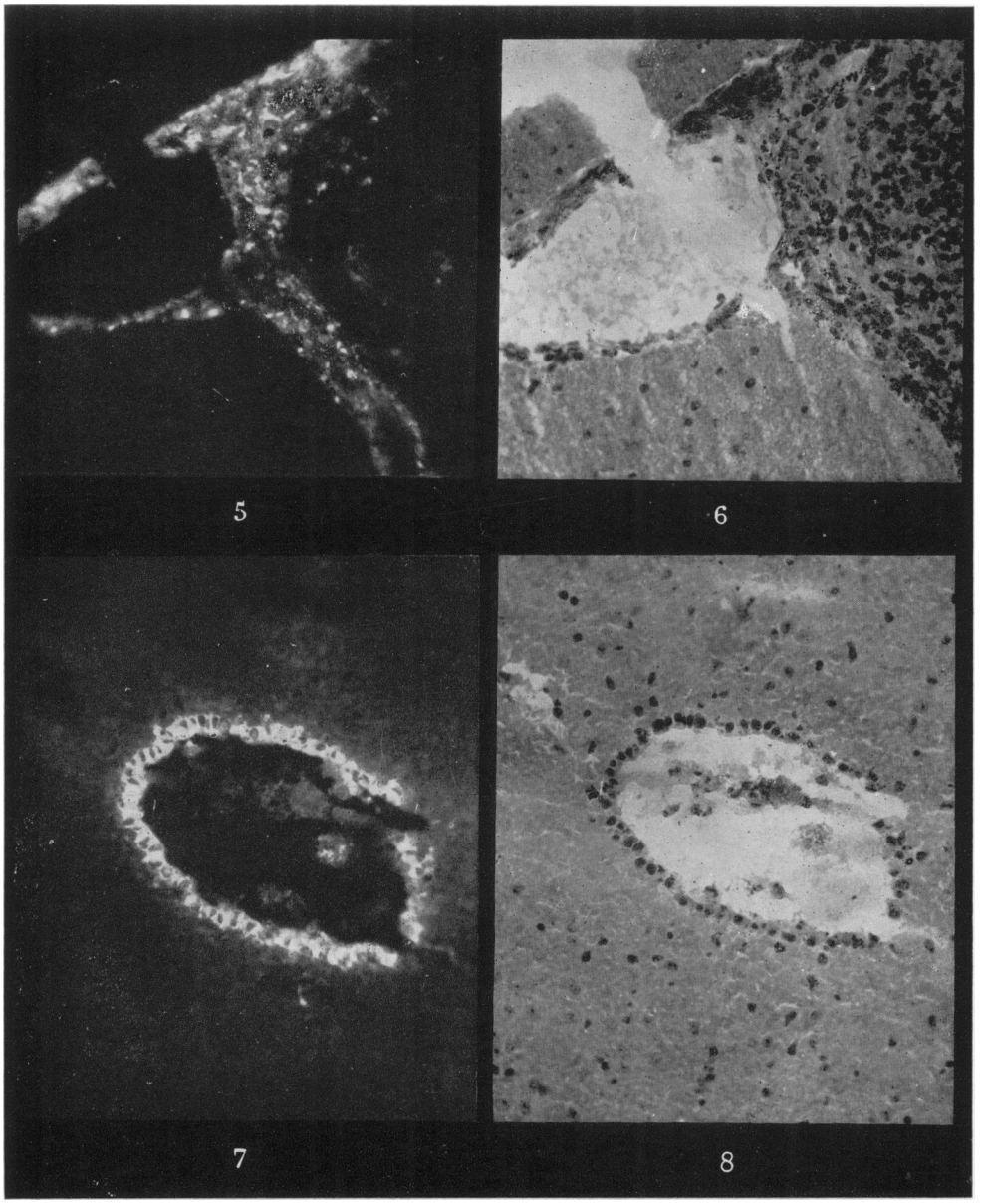

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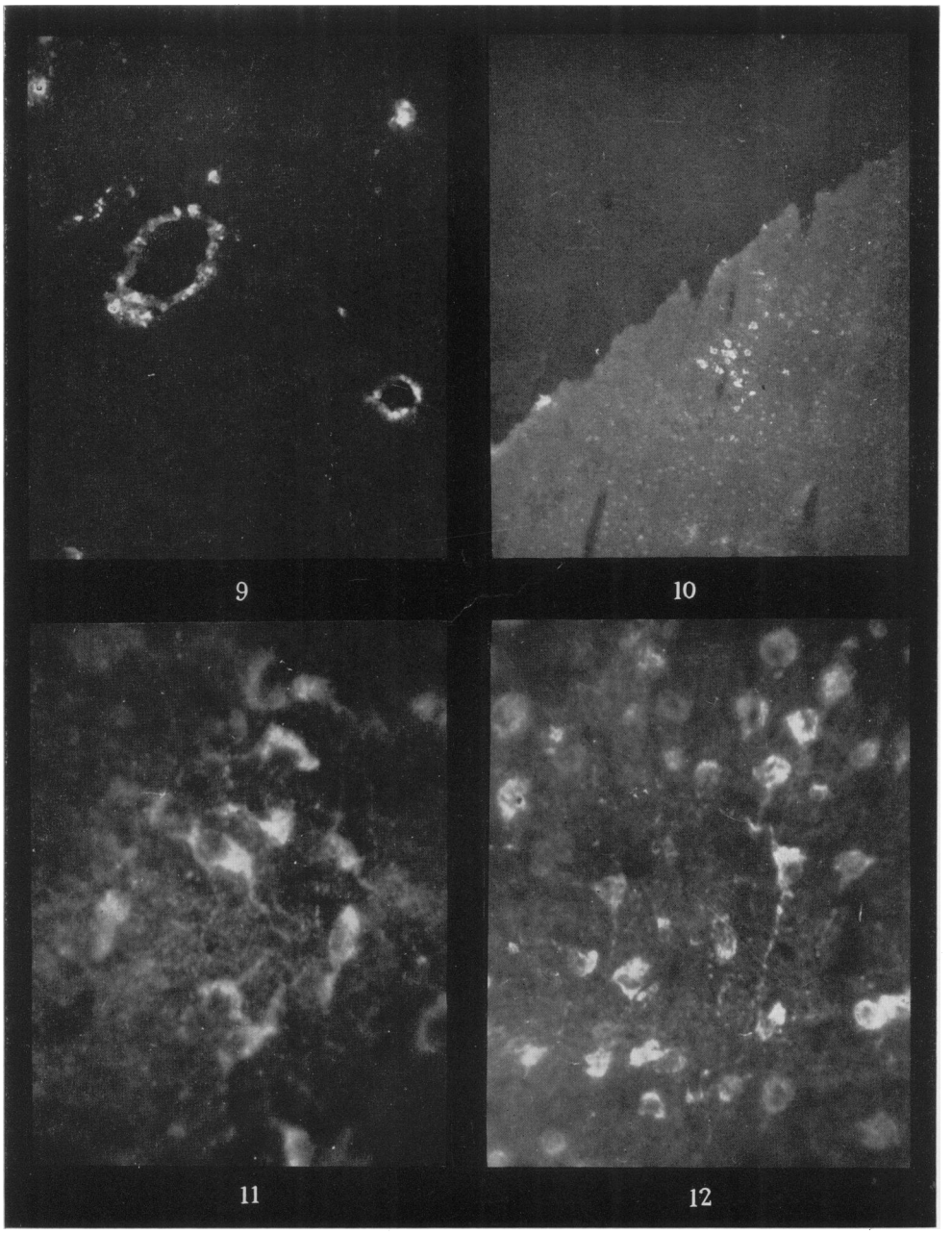

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It can be noted that viruses which grow in the continuous sheet of cells lining the CSF space, and which may kill the mouse when a large proportion of these cells have been involved, can be lethal within a short time. Viruses which grow in neurones or neuroglia, however, have to spread through a complex three-dimensional cell system after each cycle of growth. The ground substance of the brain (neuropil), far from being an extracellular fluid permitting the free diffusion of virus through it, is in fact a complex system of cytoplasmic processes from other cells (Palay, 1958). Foci enlarge irregularly as virus spreads from infected cells to those in contact with them or with their processes, until certain regions of the brain are lethally damaged, for it is probably growth in certain important regions of the brain, rather than the total amount of growth, which proves fatal. Thus, although a large intracerebral injection of rabbitpox virus kills mice in 2 days, the largest dose of MVE virus which can be given does not kill until the fourth day.

The fluorescent antibody technique was also used by Noyes (1955) and Lebrun (1956) to study the growth of viruses in the mouse brain. Noyes showed that West Nile virus, closely related to MVE virus, grew in neurones, while Lebrun concluded that Herpes Simplex virus grew in glial and ependymal cells, but not in neurones.

SUMMARY

Very high pressures are needed to inject mice intracerebrally, and large volumes are usually injected. Some of the injected material is deposited in the CSF spaces in the brain, but the high injection pressure breaks down the CSF-blood barrier, so that the rest spills over into the blood.

The fluorescent antibody technique has been used to identify the cells in the mouse brain which support the growth of certain intracerebrally injected viruses.

Intracerebrally injected ectromelia and rabbitpox viruses grow in the cells lining the cerebrospinal fluid spaces to produce meningitis and ependymitis. There is little or no growth in the brain parenchyma.

Intracerebrally injected Murray Valley Encephalitis virus grows in neurones or neuroglia, and not in cells lining the CSF spaces. Its passage across the CSF-brain barrier has been investigated.

It is a pleasure to acknowledge the competent histological assistance of John Benge.

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